ELSEVIER

Journal of Chromatography A, 767 (1997) 77–85

JOURNAL OF CHROMATOGRAPHY A

Analysis of bioactive peptides by liquid chromatography–high-resolution electrospray mass spectrometry

P.A. D'Agostino*, J.R. Hancock, L.R. Provost

Defence Research Establishment Suffield, P.O. Box 4000, Medicine Hat, Alberta T1A 8K6, Canada

Received 19 September 1996; revised 10 December 1996; accepted 10 December 1996

Abstract

LC–high-resolution electrospray ionization (ESI)-MS data for a number of bioactive peptides, including substance P and bradykinins were acquired over a wide mass range by scanning the magnetic sector and calibrating externally with polyethylene glycol standards. Multiply charged ions were observed and errors between observed and theoretical monoisotopic molecular masses were typically in the 5 to 30 ppm range for the peptides during LC–ESI-MS and ESI-MS operation with magnetic sector resolutions between 2500 and 6000 (10% valley definition). Under collisionally activated dissociation conditions b_n- and y_n-series sequence ions were generally observed, enabling amino acid sequencing and the differentiation of lysine from glutamine, two amino acids differing in residue mass by only 0.0364 u. Mass accuracy was evaluated during an international round robin analytical exercise where the molecular masses of five unknown peptides were to be accurately determined. Isotopic clusters for charge states of up to +6 were fully resolved, facilitating the rapid and unambiguous assignment of charge states and calculation of monoisotopic molecular masses. Errors between theoretical and observed monoisotopic molecular masses were in the 2 to 18 ppm range for the five unknown peptides.

Keywords: Molecular mass determination; Detection, LC; Peptides; Bradykinins; Substance P

1. Introduction

Barber et al. revolutionized mass spectrometry (MS) in the early 1980s by demonstrating the use of fast atom bombardment (FAB) MS for the analysis of peptides [1]. In the ensuing years, the useful mass range of MS for biomolecule applications increased, particularly with the demonstration by Fenn and co-workers that electrospray ionization (ESI) could be used to form multiply charged gaseous ions from large biomolecules [2,3]. Biemann [4] has reviewed the MS of peptides and proteins and the current status of biological MS was recently reviewed by Burlingame et al. [5]. Numerous references, reviews and books are cited in these reviews and they serve as a good starting point for researchers interested in assessing the potential of biological MS.

ESI was initially interfaced to a quadrupole mass spectrometer [2,3] and most of the applications in the literature deal with this type of instrumentation. Many users in the MS community conduct research with higher-resolution instruments, in large part because of the accurate mass measurement capabilities of these instruments. High-resolution data has been collected following ESI introduction of biomolecules into Fourier transform mass spectrometers and this topic was recently reviewed by Buchanan and Hettich [6]. Di- or tri-sector geometry mass

*Corresponding author.

0021-9673/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(97)00017-4

spectrometers, although not capable of the resolution of Fourier transform mass spectrometers, are more common and have been used extensively for the acquisition of high-resolution data.

Development of suitable ESI interfaces for high-resolution magnetic sector use [7] was in large part driven by the potential to increase mass measurement accuracy. Use of high-resolution enables the assignment of charge state to multiply charged isotope clusters and aids in the interpretation of amino acid sequence data during collisionally activated dissociation (CAD) in the region between the capillary exit and skimmer in the ESI interface [8–15]. Particularly valuable is the ability to determine monoisotopic molecular mass, as these values are independent of $^{12}C/^{13}C$ variations.

Relatively low-resolution CAD-MS spectra, yielding characteristic a_n-, b_n- and y_n-series ions, have been acquired in the region between the capillary exit and the skimmer region in an ESI interface at a resolution of 1000 to 1500 (10% valley definition) for three model peptides [8]. Starrett and DiDonato, working at a resolution of 5000 (5% valley definition) under voltage scanning conditions over a narrow mass range concentrated on the accurate mass measurement of product ions generated during CAD-MS [14]. Use of an internal calibrant minimized differences between the theoretical and observed product ion masses to about 5 ppm for several peptides including angiotensin II and substance P (fragment 1–9). In one case the full primary sequence of a peptide, human renin substrate, was determined with the exception that the leucine and isoleucine isomers could not be differentiated [8]. The value of high-resolution for the assignment of charge state for CAD-MS product ions was demonstrated by Loo et al. in a paper focusing on the determination of protein structural information following ESI introduction [13].

Larsen and McEwen [9] employed resolutions of 5000 and 10 000 (10% valley definition) for accurate molecular mass determination and found that errors seldom exceeded 25 ppm for several pure peptides. Calibration was done internally and the isotopic cluster for the +5 charge state of insulin was resolved. High resolution separation of a $(M+9H)^{9+}$ isotopic cluster was demonstrated by Cody et al. for lysozyme at resolution of 10 000 (10% valley). Errors associated with these measurements were in the 5 to 20 ppm range when internal calibration was employed [12]. Higher errors, typically in the 5 to 90 ppm range, were observed when external calibration was used over a 10 h period.

The molecular masses for a series of thirty-seven unknown synthetic peptides, used in research studies involving synthetic vaccines, antibacterial peptides or the de novo design of helical peptides and proteins, were determined with a magnetic sector instrument [15]. All data were obtained with external calibration over a wide mass range during magnetic scanning. Errors between observed and theoretical monoisotopic molecular masses were typically in the 5 to 60 ppm range for the unknowns at sector resolutions between 2500 and 9000 (10% valley). Isotopic clusters for charge states up to +10 were resolved through the use of high resolution.

Bioactive peptides, including bradykinins and substance P, have been analysed frequently by ESI-MS during demonstration of instrument performance [16]. Most of this effort has involved the use of quadrupole instrumentation with no emphasis on the use of liquid chromatography (LC) prior to high-resolution ESI-MS analysis with a magnetic sector instrument [5]. LC–ESI-MS and ESI-MS data for a number of bioactive and unknown peptides were acquired over a wide mass range by scanning the magnetic sector and calibrating externally with polyethylene glycol standards. Errors between observed and theoretical monoisotopic molecular masses were typically in the 5 to 30 ppm range for bioactive and unknown peptides with magnetic sector resolutions between 2500 and 6000 (10% valley definition). Isotopic clusters for charge states of up to +6 were fully resolved. Under CAD-MS conditions both b_n- and y_n-series sequence ions were generally observed, enabling amino acid sequencing of peptides or their tryptic fragments.

2. Experimental

2.1. Samples and sample handling

All the bioactive peptides were purchased from Sigma (St. Louis, MO, USA). Each peptide was dissolved to an initial concentration of 0.3 to 1

102-02584/

mg/ml in distilled water or distilled water containing 0.05% HPLC grade trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, USA). Standard solutions were stored at −20°C and diluted by a factor of 10 with distilled water prior to analysis. Further dilutions were made to estimate detection limits. Distilled-in-glass water was filtered through a 0.45 μm Millipore filter prior to use in the mobile phase or for diluting the peptide samples. Acetonitrile was Burdick and Jackson UV grade (Muskegon, MI, USA).

Tryptic digests were prepared by dissolving modified sequencing grade trypsin (Boehringer Mannheim, Mannheim, Germany) in 0.1 *M* ammonium bicarbonate and adding this trypsin solution (50 μl of 0.1 mg/ml) to the peptide (200 μl of 0.5 mg/ml), giving an enzyme–substrate ratio of about 1:20. Digestions were carried out for about 18 h at 37°C and were quenched with TFA (to pH 2–3) prior to LC–ESI-MS analyses.

2.2. International round robin analytical exercise

The chemical/biological defence community has an interest in determining peptide or protein content in samples or cell extracts and organized an international round robin analytical exercise to assess MS methods for unknown peptide molecular mass determination. Each of seven laboratories received five unknown peptides that were synthesized and distributed by Dr. R. Hodges' Group (Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Canada) as lyophilized powders. All the peptides were in the 400 to 5000 u mass range, simulating the masses of peptides that may be encountered during analysis of samples for bioactive peptides.

2.3. Instrumental

All ESI mass spectra were acquired using a Micromass Autospec-Q mass spectrometer (Manchester, UK) equipped with the Mark II ESI interface. The electrospray needle was operated at 7.6 kV and ions were accelerated into the mass spectrometer at 4 kV. Sampling cone voltages up to 175 V were initially investigated, with all subsequent data being acquired with sampling cone voltages in the 75 to 150 V range. Nitrogen (Very Dry, Liquid Carbonic Inc., Scarborough, Canada) bath gas was introduced into the interface (80°C) at a flow-rate of 500 l/h. Nitrogen nebulizer gas was introduced at a flow-rate of 14 l/h. The ESI interface was pumped with both a rotary and a turbomolecular pump, which enabled maintenance of $4 \cdot 10^{-4}$ and $7 \cdot 10^{-6}$ Pa within the source and analyser regions, respectively.

ESI data were acquired in the continuum mode by scanning the magnet over a variety of mass ranges such that each scan took about 6–8 s. This resulted in the acquisition of five to ten scans for each sample component during LC–ESI-MS analysis. Resolutions of 2500 to 6000 (10% valley definition) were employed during magnetic sector scanning to facilitate accurate mass measurement of the ions formed during ESI-MS analyses. External calibrations were performed with solutions of polyethylene glycol in distilled water. Monoisotopic molecular masses for the bioactive peptides were calculated in triplicate from the observed $(M+nH)^{n+}$ ions, where the charge states were determined by the isotopic cluster spacings.

ESI-MS–MS data were acquired with a quadrupole CAD cell energy of 100 V and an argon pressure of about $1.3 \cdot 10^{-2}$ Pa near the CAD cell. These conditions attenuated the precursor ions by about 70% and represented a good compromise between sensitivity and spectral content for these analyses. Precursor ions were selected with a magnetic sector resolution of 1000 (10% valley definition) and product ions were detected by scanning the quadrupole from 600 to 80 u.

HPLC separations of the bioactive peptides were performed with an Applied Biosystems Model 140B dual syringe pump (Foster City, CA, USA) equipped with a 15 cm×0.53 mm I.D. C_{18} (5 μm) packed J and W DB-1 fused-silica capillary column (courtesy of Mr. L. Hogge and Mr. D. Olson, NRC, Saskatoon, Canada). The following solvent compositions were prepared for ESI-MS sample introduction: solvent A (0.05% TFA in water) and Solvent B (0.05% TFA in acetonitrile–water, 80:20). Chromatographic separations were performed using a 5% to 65% B linear gradient over 30 or 60 min. In order to minimize dead volume and ensure reproducible mixing, the mobile phase was delivered at 200 μl/min and split prior to the injector such that the flow through the

column was 20 μl/min. Loop injections (20 μl) were made with 50% B.

3. Results and discussion

The ESI-MS data of many peptides and proteins have been acquired under low-resolution conditions with quadrupole instruments, with relatively few examples of high-resolution mass measurement with magnetic sector instruments [8–15]. Of those reported, none appear to have been obtained following on-line separation by LC or other separatory techniques [5]. A prior study indicated the value of high-resolution mass measurement for monoisotopic molecular mass determination, charge assignment of $(M+nH)^{n+}$ clusters and, perhaps most importantly, for the acquisition of amino acid sequence information during CAD-MS analyses [15]. Demonstration of high-resolution ESI-MS data acquisition for bioactive peptides and their tryptic fragments following LC separations would be of considerable value, since analysts are generally confronted with multicomponent samples. Improved accurate mass measurement would reduce the number of possible matches during peptide database searching and would facilitate more complete amino acid sequencing.

3.1. Monoisotopic molecular mass determinations

About a dozen commercially available bioactive peptides were analysed by LC–high-resolution-ESI-MS under sampling cone conditions that generally favor observation of isotopic cluster ions with little or no product ion formation. In general $(M+H)^+$ isotopic clusters were most significant for lower-molecular-mass peptides. Higher-molecular-mass peptides typically stabilize more charge and tend to form isotopic clusters of higher charge state, provided sufficient basic sites are available for protonation. The chromatogram illustrated in Fig. 1 was typical of a gradient separation (1% B/min) of a mixture of bioactive peptides. The acquired ESI-MS data for the three bradykinins contained $(M+nH)^{n+}$ isotopic clusters from which the monoisotopic molecular mass may be calculated. The monoisotopic molecular mass for each peptide was determined in

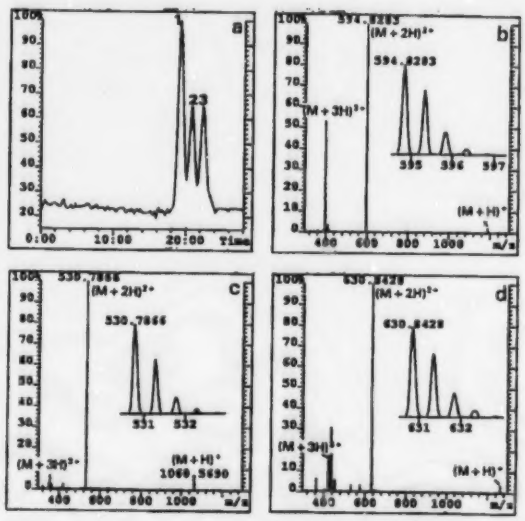

Fig. 1. (a) Capillary column LC–ESI-MS total-ion-current (1300 to 300 u) chromatogram for Lys–bradykinin (1), bradykinin (2) and Ile–Ser–bradykinin (3) with a magnetic sector resolution of 3000 and a sampling cone voltage of 75 V. ESI-MS data for: (b) Lys–bradykinin, calculated molecular mass 1187.64±0.02 (n=3), 13 ppm error; (c) bradykinin, calculated molecular mass 1059.559±0.003 (n=3), 2 ppm error and (d) Ile–Ser–bradykinin, calculated molecular mass 1259.67±0.03 (n=3), 5 ppm error.

triplicate using the ^{12}C ion in each of the +1, +2 and +3 isotopic clusters. Mass accuracy errors were in the 2 to 13 ppm range during LC–ESI-MS analysis with a magnetic sector resolution of 3000, typical of errors that have been obtained during ESI-MS operation [15].

Detection limits during LC–high resolution-ESI-MS were estimated for two bioactive peptides, Ile–Ser–bradykinin and substance P, at the 20 pmol level (Fig. 2). In each case the signal-to-noise ratio (S/N) was in excess of 50:1, with calculated monoisotopic molecular masses being within 15 ppm of theoretical values. Based on this data a detection limit of 1 pmol (S/N=5:1) was estimated. Similar detection limits were found for other bioactive peptides.

3.2. LC–ESI-MS of Met–Lys–bradykinin tryptic digestion

The ESI-MS data acquired for Met–Lys–bradykinin with a sampling cone voltage of 125 V

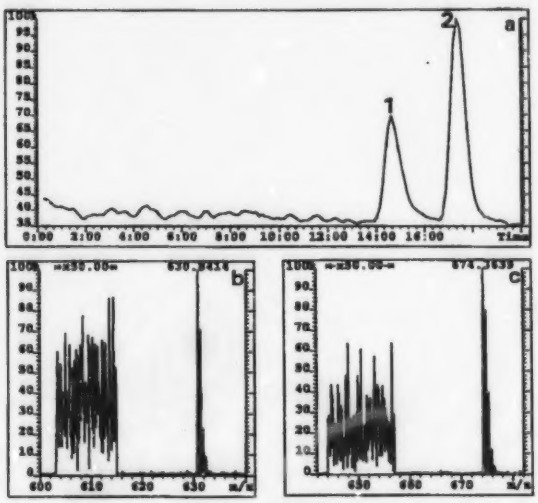

Fig. 2. (a) Capillary column LC–ESI-MS total-ion-current (720 to 600 u) chromatogram for 20 pmol of Ile–Ser–bradykinin (1) and substance P (2) with a magnetic sector resolution of 2500 and a sampling cone voltage of 75 V. *S/N* was greater than 50:1 for: (b) Ile–Ser–bradykinin (calculated molecular mass 1259.667, 8 ppm error) and (c) substance P (calculated molecular mass 1346.712, 12 ppm error).

provided monoisotopic molecular mass data in good agreement with theoretical data (Table 1), but the product ion relative intensities were too low for complete amino acid sequencing of the intact peptide. Tryptic digestion was performed to provide smaller peptide fragments that could give rise to more intense product ions during CAD-MS. Three tryptic fragments, two of which were predicted by cleavage at the C-terminus of the lysine residue,

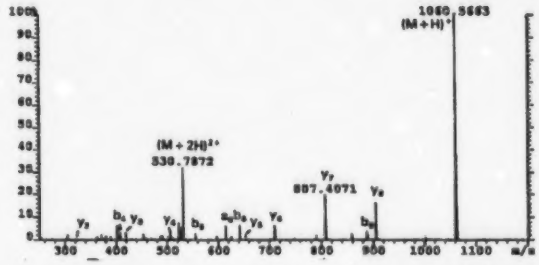

Fig. 3. CAD-MS data for Met–Lys–bradykinin tryptic fragment 2 (RPPGFSPFR) obtained following capillary column LC–ESI-MS analysis with a magnetic sector resolution of 3000 and a sampling cone voltage of 150 V (Refer to Table 2).

were observed. The third component was identified following amino acid sequencing under CAD-MS conditions.

The monoisotopic molecular masses of the three tryptic fragments were within 25 ppm of predicted values (Table 1) and the monoisotopic molecular mass of tryptic fragment 3 suggested the presence of an unexpected cleavage of an arginine amino acid residue from either the N- or C-terminus. Under CAD-MS conditions, with a sampling cone voltage of 150 V, it was possible to obtain both the molecular mass and the complete amino acid sequence of tryptic fragment 2 (Fig. 3). Overlap of the y_n- and b_n-series was observed and amino acid residue losses, typically within 0.008±0.006 u (n=11) of those predicted, were observed for this tryptic fragment (Table 2). The CAD-MS data for tryptic fragment 3 were less informative but the presence of a_6 (m/z 614.34) and a_5 (m/z 527.32) ions were

Table 1
Electrospray MS data obtained for Met–Lys–bradykinin and the tryptic fragments of Met–Lys–bradykinin during LC–MS analysis

Peptide structure (primary sequence)	Observed monoisotopic molecular mass*	Theoretical monoisotopic molecular mass	Error (ppm)
Met–Lys–bradykinin (MKRPPGFSPFR)	1318.701±0.007 (n=3)	1318.6968	3
Tryptic fragment 1 (MK)	278.1470	278.1538	24
Tryptic fragment 2 (RPPGFSPFR)	1059.561±0.013 (n=3)	1059.5614	0
Tryptic fragment 3 (RPPGFSPF)	903.472±0.008 (n=2)	903.4603	13

* Average of $(M+H)^+$, $(M+2H)^{2+}$ and/or $(M+3H)^{3+}$ data. Data were obtained at a magnetic sector resolution of 3000 and a sampling cone voltage of 75 V.

Table 2
CAD-MS data obtained for tryptic fragment 2[a] of Met–Lys–bradykinin during LC–MS analysis

Series	m/z	Amino acid residue	Theoretical residue	Theoretical residue mass	Observed residue mass
y_8	904.4618	Arg (R)	156.10111	156.1045	0.003
y_7	807.4071	Pro (P)	97.05276	97.0547	0.002
y_6	710.3468	Pro (P)	97.05276	97.0603	0.008
y_5	653.3364	Gly (G)	57.02146	57.0104	0.011
y_4	506.2741	Phe (F)	147.06841	147.0623	0.006
y_3	419.2448	Ser (S)	87.03203	87.0293	0.003
y_2	322.1749	Pro (P)	97.05276	97.0699	0.017
b_8	886.4427	Arg (R)+H_2O	174.11168	174.1236	0.012
b_6	642.3392	Phe (F)+ Pro (P)	244.12117	244.1035	0.018
b_5	555.3049	Ser (S)	87.03203	87.0343	0.002
b_4	408.2328	Phe (F)	147.06841	147.0721	0.004

[a] Data were obtained by scanning from 1200 to 250 u (8 s/decade) at a magnetic sector resolution of 3000 and a sampling cone voltage of 150 V.

indicative of loss of proline and phenylalanine and loss of serine, proline and phenylalanine, respectively, from the C-terminus. This was sufficient to confirm that the difference between tryptic fragments 2 and 3 was due to the loss of an arginine from the C-terminus of tryptic fragment 2.

3.3. International round robin analytical exercise

Seven laboratories, including one in Canada, recently participated in an international training exercise designed to evaluate the capabilities of each laboratory to determine the molecular masses of five unknown peptides with molecular masses in the same mass range as bioactive peptides of interest to the chemical/biological defence community. Table 3 lists the amino acid sequences and monoisotopic and average molecular masses for each of the five unknown peptides labelled A to E.

The accurate determination of molecular mass is considered to be the initial step in the identification of an unknown peptide. All the participating laboratories demonstrated the basic ability to determine either monoisotopic or average molecular masses for the unknown peptide samples by ESI-MS (Table 4). The most accurate results, with an average ppm error of 9.2±6.6 ppm, were reported by Canada using ESI interfaced to a high-resolution mass spectrometer. Canadian data were acquired following loop injections of each unknown peptide. The monoisotopic molecular masses were first estimated by magnetic scanning over a wide mass range (e.g., 825 to 110 u or 1400 to 400 u) at resolutions between 3000 and 6000 (10% valley definition). The mass range was then narrowed for more accurate calculation of monoisotopic molecular masses (e.g., 570 to 440 u or 790 to 660 u). Figs. 4a and 5a illustrate ESI-MS data obtained for peptides A and E, respectively. In all cases the $(M+nH)^{n+}$ isotopic clusters were well resolved, enabling immediate charge state assign-

Table 3
Amino acid sequences and molecular masses for unknown peptides

Peptide	Amino acid sequence	Monoisotopic molecular mass	Average molecular mass
A	FIPK	503.310755	503.642077
B	AGKDYDKIEE	1166.545505	1167.237777
C	ATKKEVPLGVAADANKLG	1781.004645	1782.070277
D	RFEMFRELNEALELKDAQAGKE-NH_2	2622.322369	2623.966791
E	VEHYDNIEQKIDDIDHEIADLQAKITRLVQQHPRIDE	4436.235975	4438.877777

Table 4
Monoisotopic and average molecular masses reported by participating laboratories during ESI-MS analyses

Laboratory (instrument type)	Peptide A		Peptide B		Peptide C		Peptide D		Peptide E		Average ppm error (n=5)
	M.M.	Error (ppm)	M.M.	Error (ppm)	M.M.	Error (ppm)	M.M.	Error (ppm)	M.M.	Error (ppm)	
Canada (Sector)	503.312±0.001[a] (n=3)	2.5	1166.550±0.006[a] (n=3)	3.9	1780.991±0.006[a] (n=3)	7.7	2622.276±0.005[a] (n=3)	18	4436.172±0.034[a] (n=4)	14	9.2±6.6
A (Ion trap)	503[a]	617	1166[a]	468	1782[a]	559	2622[a]	123	4437[a]	172	387±226
B (Quadrupole)	503.4[a]	177	1166.6[a]	47	1781.2[a]	110	2623.8	64	4438.6	63	92±53
C (Sector)	503[a]	617	1167[a]	390	1781	601	2623	368	4439	28	401±238
D (Quadrupole)	503.4	481	1166.7	461	1781.4	376	2623.8	64	4438.2	153	307±188
E (Quadrupole)	503.9	512	1167.6	310	1784.6	1420	2624.2	89	4438.4	108	488±549
F (Quadrupole)	503.4[a]	177	1166.7[a]	132	1781.3[a]	166	2622.8[a]	182	4437.7	265	184±49

[a] Monoisotopic molecular mass.

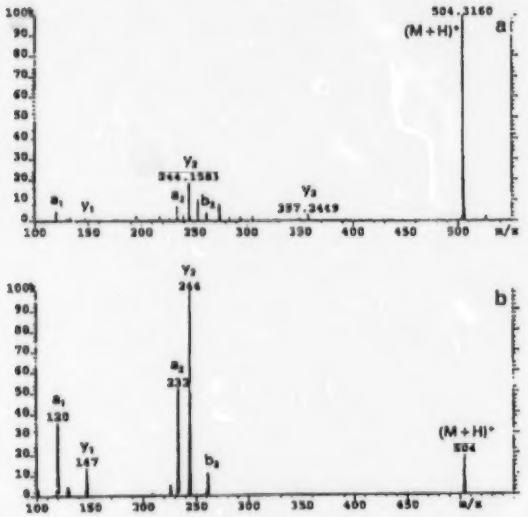

Fig. 4. (a) CAD-MS (75 V) and (b) MS–MS (quadrupole CAD cell; 100 V, 1.4×10^{-3} Pa Argon) data obtained during analysis of peptide A.

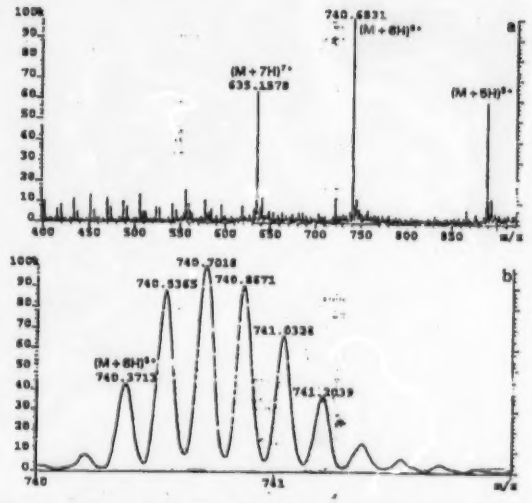

Fig. 5. (a) High-resolution ESI-MS data acquired for peptide E during magnetic sector scanning over a wide mass range [925 to 400 u]. (b) $(M+6H)^{6+}$ isotopic cluster acquired during magnetic sector scanning over a narrow mass range [790 to 660 u]. MS conditions: sampling cone voltage, 25 V; resolution, 6000 (10% valley). Calculated monoisotopic molecular mass: 4436.172±0.034 (n=4).

ment as well as calculation of monoisotopic molecular mass from the ^{12}C containing ion in the isotopic cluster.

The amino acid sequence for unknown peptide A was assigned following interpretation of product spectra generated following CAD-MS and MS–MS of the $(M+H)^+$ ion at m/z 504 (Fig. 4). Table 5 lists the ions observed and the cleavages for unknown peptide A, identified as Phe–Ile/Leu–Pro–Lys as opposed to Phe–Ile/Leu–Pro–Gln based on high resolution ESI-CAD-MS data. High-resolution data could not be used to differentiate between Ile and

Table 5
Ions observed for unknown peptide A: Phe–Ile/Leu–Pro–Lys

Ion observed	CAD-MS					MS–MS
	m/z	Residue(s) cleaved	Observed mass loss	Theoretical mass loss	Error (u)	
$(M+H)^+$	504.3160				0.003	504
	(504.2822)				(0.034)	
y_3	357.2449	Phe	147.0711	147.0684	0.003	357
y_2	244.1583	Phe+Ile/Leu	260.1577	260.1525	0.005	244
y_1	147.1046	Phe+Ile/Leu+Pro	357.2114	357.2052	0.006	147
	(147.0770)				(0.028)	
b_2	261.1411	Lys+Pro+H_2O	243.1749	243.1583	0.017	261
		(Gln+Pro+H_2O)	(243.1749)	(243.1219)	(0.053)	
a_2	233.1585	Lys+Pro+CH_2O_2	271.1575	271.1532	0.004	233
		(Gln+Pro+CH_2O_2)	(271.1575)	(271.1168)	(0.041)	
a_1	120.0510[a]	Lys+Pro+Ile/Leu+CH_2O_2	384.2650	384.2373	0.028	120
		(Gln+Pro+Ile or Leu+CH_2O_2)	(384.2650)	(384.2009)	(0.064)	

Values in parentheses represent theoretical masses and errors for Phe–Ile/Leu–Pro–Gln.
[a] Mass measurement error larger since the m/z ratio was below the lowest external calibration mass.

Leu but enabled the differentiation of lysine from glutamine, two amino acid residues that differ in mass by only 0.0364 u. The $(M+H)^+$ and y_1 ions differed from the theoretical masses by 0.003 and 0.006 u, respectively, at a magnetic sector resolution of 3000. Similar errors were observed for the b_2 and a_2 ions. In all cases the values in parentheses in Table 5, which indicate the mass and errors that would be associated with glutamine incorporation, were considerably larger (>0.03 u). These data clearly indicated lysine, as opposed to a glutamine, for the C-terminus of unknown peptide A.

The value of high-resolution was further demonstrated by the analysis of unknown peptide E with a magnetic sector resolution of 6000. Charge states for the isotopic clusters could be determined immediately based on mass spacings and the monoisotopic molecular mass could be accurately determined from the ion containing only ^{12}C. Fig. 5 illustrates the acquired ESI-MS data for this peptide, including an expansion of the completely resolved $(M+6H)^{6+}$ isotopic cluster.

4. Conclusions

Considerable effort has gone into the ESI-MS analysis of bioactive peptides by a number of research groups, but relatively little effort has been directed towards the identification of these compounds under high-resolution conditions with a magnetic sector mass spectrometer. Past efforts have focussed on the use of loop injection with no emphasis on chromatographic separation prior to ESI-MS analysis under high-resolution conditions with magnetic sector instruments.

LC–high-resolution-ESI-MS data for a number of bioactive peptides, including substance P and bradykinins were acquired over a wide mass range by scanning the magnetic sector and calibrating externally with polyethylene glycol standards. Multiply charged ions were observed and errors between observed and theoretical monoisotopic molecular masses were typically in the 5 to 30 ppm range for the peptides during LC–ESI-MS and ESI-MS operation with magnetic sector resolutions between 2500 and 6000 (10% valley definition). Under CAD-MS conditions both b_n- and y_n-series sequence ions were generally observed, enabling amino acid sequencing of the peptide fragments generated by tryptic digestion.

High-resolution ESI-MS methods were evaluated during an international round robin analytical exercise where the molecular masses of five unknown peptides were to be accurately determined. Isotopic clusters for charge states of up to +6 were fully resolved, facilitating the rapid and unambiguous assignment of charge states and calculation of monoisotopic molecular masses. The value of high-resolution ESI-CAD-MS analysis was further demonstrated by differentiation of lysine from glutamine, two amino acids differing in residue mass by only 0.0364 u. Errors between theoretical and observed monoisotopic molecular masses were in the 2 to 18 ppm range for the five unknown peptides, the same range as those observed during LC–ESI-MS analysis of bioactive peptides.

References

[1] M. Barber, R.S. Bordoli, R.D. Sedgwick and A.N. Tyler, J. Chem. Soc. Chem. Commun., (1981) 325.
[2] C.K. Meng, M. Mann and J.B. Fenn, Z. Phys. D, 10 (1988) 361.
[3] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, Science, 246 (1989) 64.
[4] K. Biemann, Annu. Rev. Biochem., 61 (1992) 977.
[5] A.L. Burlingame, R.K. Boyd and S.J. Gaskell, Anal. Chem., 66 (1994) 634R.
[6] M.V. Buchanan and R.L. Hettich, Anal. Chem., 65 (1993) 245A.
[7] C.K. Meng, C.N. McEwen and B.S. Larsen, Rapid Commun. Mass Spectrom., 4 (1990) 147.
[8] C.K. Meng, C.N. McEwen and B.S. Larsen, Rapid Commun. Mass Spectrom., 4 (1990) 151.
[9] B.S. Larsen and C.N. McEwen, J. Am. Soc. Mass Spectrom., 2 (1991) 205.
[10] J.R. Chapman, R.T. Gallagher, E.C. Barton, J.M. Curtis and P.J. Derrick, Org. Mass Spectrom., 27 (1992) 195.
[11] C.N. McEwen and B.S. Larsen, Rapid Commun. Mass Spectrom., 6 (1992) 173.
[12] R.B. Cody, J. Tamura and B.D. Musselman, Anal. Chem., 64 (1992) 1561.
[13] J.A. Loo, R.R. Ogorzalek Loo and P.C. Andrews, Org. Mass Spectrom., 28 (1993) 1640.
[14] A.M. Starrett and G.C. DiDonato, Rapid Commun. Mass Spectrom., 7 (1993) 12.
[15] P.A. D'Agostino, J.R. Hancock, L.R. Provost, P.D. Semchuk and R.S. Hodges, Rapid Commun. Mass Spectrom., 9 (1995) 597.
[16] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga and H.R. Udseth, Anal. Chem., 62 (1990) 882.